Journal of the Formosan Medical Association (2014) 113, 42—49

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ORIGINAL ARTICLE

Multiphoton microscopy imaging of developing tooth germs

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Received 20 January 2012; received in revised form 20 March 2012; accepted 28 March 2012

KEYWORDS
autofluorescence;
dentistry;
multiphoton laser fluorescence microscope;
second-harmonic generation signal;
tooth germ

Background/Purpose: Traditionally, tooth germ is observed by histological investigation with hematoxylin and eosin stain and information may loss during the process. The purpose of this study is to use multiphoton laser fluorescence microscopy to observe the developing tooth germs of mice for building up the database of the images of tooth germs and compare with those from conventional histological analysis.
Methods: Tooth germs were isolated from embryonic and newborn mice with age of Embryonic Day 14.5 and Postnatal Days 1, 3, 5, and 7.
Results: Comparison of the images of tooth germ sections in multiphoton microscopy with the images of histology was performed for investigating the molar tooth germs. It was found that various signals arose from different structures of tooth germs. Pre-dentin and dentin have strong second-harmonic generation signals, while ameloblasts and enamel tissues were shown with strong autofluorescence signals.
Conclusion: In this study, a novel multiphoton microscopy database of images from developing tooth germs in mice was set up. We confirmed that multiphoton laser microscopy is a powerful tool for investigating the development of tooth germ and is worthy for further application in the study of tooth regeneration.

The authors have no conflicts of interest relevant to this article.

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0929-6646/$ - see front matter
doi:10.1016/j.jfma.2012.03.016

Introduction

In 1990, Denk and colleagues[1] developed the foundation of multiphoton fluorescence microscopy. It is widely used in biomedical research ever since then.[1] Multiphoton fluorescence excitation and multiharmonic signals are nonlinear optical phenomena. The nonlinear quality enhances the contrast between focal region and background, thus making the "optical dissection" possible. For the same reason, the multiphoton microscopy image can be acquired without dissecting and staining of the sample.

Autofluorescence (AF) from cellular constituents can provide both morphological and functional information. In addition, many non-central-symmetric biological structures can be used to produce second-harmonic generation (SHG) signals. SHG is a coherent, nonlinear process that generates particularly strong signals when the molecules are orderly arranged. Biological structures such as fibril collagen, muscle fibers, and microtubules are known to be effective in generating second-harmonic signals.

In recent years, multiphoton microscopy is applied on biomedical research, even including biomedical fundamental and clinical studies.[2–6] Campagnola and colleagues[7] used multiphoton microscopy to observe cells, tissues, and organs, while Brown and coworkers[8] used it to study the collagen fiber of tumor. Michael and colleagues[9] used it to observe brain tissue. As in dentistry, Hall and Girkin and others[10] mentioned that multiphoton would be a diagnostic tool for caries in the future. Chen and coworkers[11] observed the abnormality of enamel by using multiphoton fluorescence microscopy.

In oral embryology, the growth and development of tooth has always been a core study issue and many information and concept have been presented. In a traditional way to observe tooth germs, histological staining is the most popular method; however, it is time consuming and inconvenient. In our previous study, the structures of dentin, enamel, and periodontal ligaments were identified by AF and SHG signals, and clear images of tooth structures were observed.[12] It showed that multiphoton microscopy could be a novel equipment for dental morphogenesis to observed the development of tooth. However, the AF and SHG signals of tooth morphogenesis in oral embryology still unclear. It is important to obtain the normal images of tooth germ tissues. The purpose of this study is to use multiphoton laser fluorescence microscopy to observe the developing tooth germs of mice for building up the database of the images of tooth germs and compare with those from conventional histological analysis.

Materials and methods

Tooth germ preparation

All animal studies were performed according to a protocol approved by the Review Committee of College of Medicine, National Taiwan University. Mandibles with tooth germs were isolated from ICR mice, in the age of Embryonic Day 14.5 (E14.5) and Postnatal Day 1s, 3, 5, and 7 (P1, P3, P5, and P7, respectively).

Firstly, mandibles were removed from mice or embryos. Then, mandibles were fixed in 4% (w/v) formaldehyde and preserved at 4°C (Fig. 1). After fixation, the samples were dehydrated, paraffin imbedded, and sectioned. Parts of the slices were prepared for investigation with multiphoton microscopy and parts of the adjacent slices of the tooth germ in the same area were prepared for comparison with histological analysis by hematoxylin and eosin (H&E) staining.

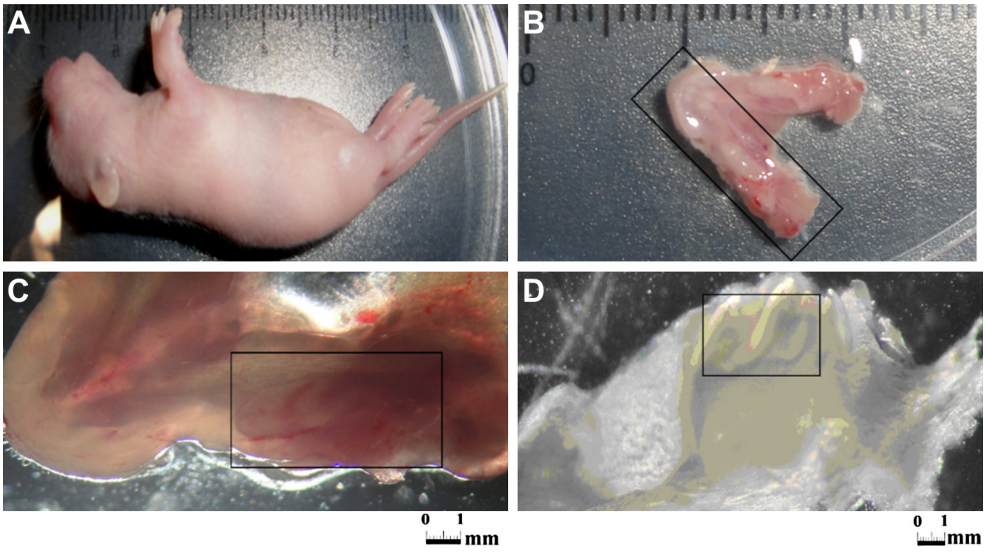

Figure 1 Mandible with tooth germs isolated from ICR mouse in the age of Postnatal Day 3. (A) Cross view of ICR mouse in the age of P3; (B) cross view of isolated mandible from ICR mouse in the age of P3; (C) cross view of mandible with tooth germ identified from the magnified image of (B); (D) tooth germs in the alveolar bone were identified under light microscopy. (The rectangle labels the sites of tooth germs.) (Scale bar: 1 mm.)

Multiphoton laser microscopy

The multiphoton AF and SHG images were acquired using a commercial laser scanning microscope (LSM 510 Meta, Zeiss, Jena, Germany) operating in non-de-scanned mode with excitation provided by an 80-MHz femtosecond Ti-sapphire laser system (Millenia X, Tsunami, Spectra Physics, Mountain View, CA, USA) tuned to 780 nm. Details of the optical setup have been described previously.[12,13] In short, the scanned laser beam passes through the main dichroic mirror (700DCXRU, Chroma Technology, Rockingham, VT, USA) and the imaging objective (C-Apochromat 40X, NA 1.2, Zeiss) on to the sample. The AF and SHG signals are collected by the same objective and further separated by a secondary dichroic mirror and two filters (440DCXR, E435LP-E700SP, HQ390/20-2p, Chroma Technology, Brattleboro, Vermont). Large area tile scanning were performed using the motorized sample stage controlled by the scanning software. In this and subsequent images, green pseudocolor was used to denote AF and the blue pseudocolor to represent SHG signal.

Results

Comparison between multiphoton microscopy images and histology images

Images of tooth germs from mice with different ages at E14.5 and P1, P3, P5, and P7 were investigated with multiphoton microscopy and histological analysis with H&E staining were compared separately.

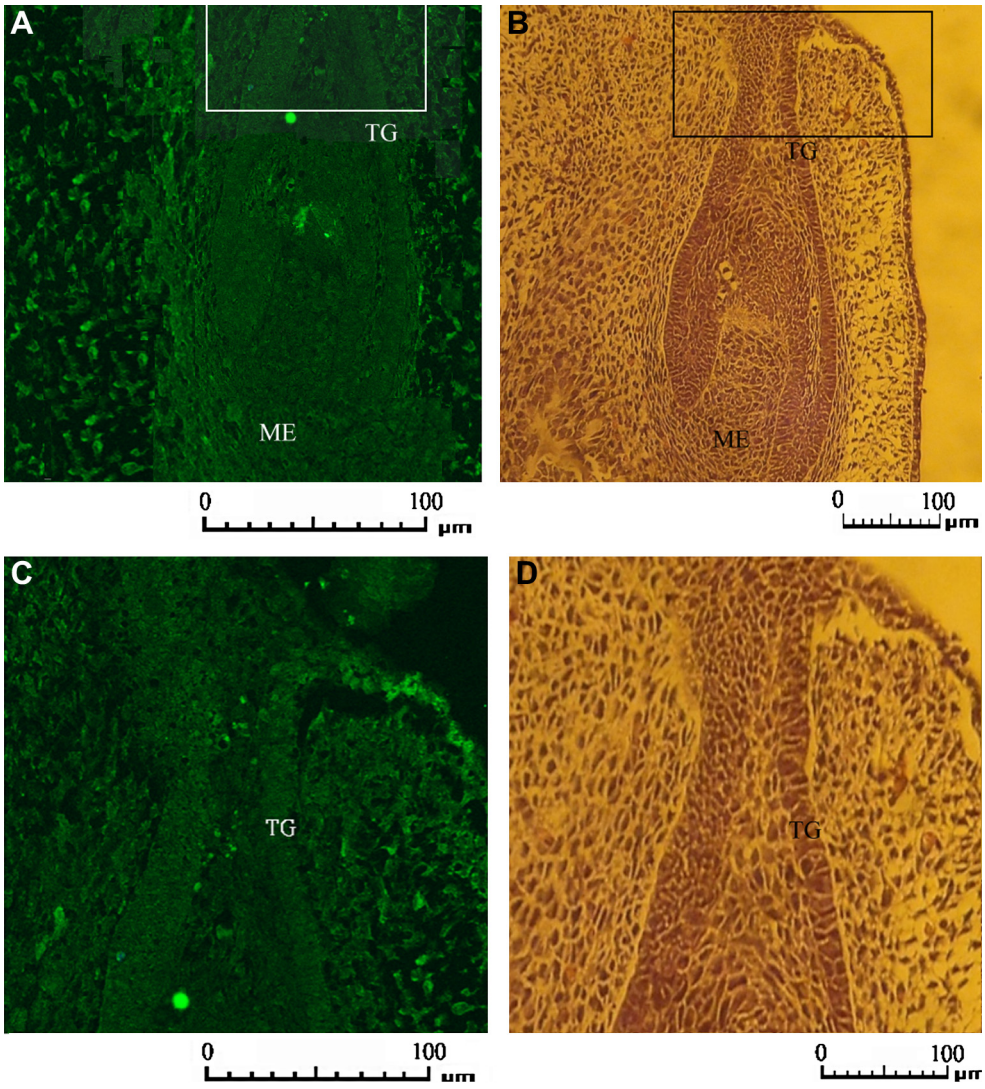

Figure 2 Comparison of images of tooth germ from E14.5 mice investigated under both multiphoton microscopy and histological analysis with hematoxylin and eosin staining observed under light microscopy. (A) Large scale of the multiphoton microscopy images revealed the expression of autofluorescence in green color in the tooth germ; (B) related histological analysis with hematoxylin and eosin staining in the same area demonstrated the connection between tooth germ and epithelium. It was found that dental epithelium cells were started to be unfolded and the mesenchymal cells were aggregated to form a dental papilla; (C) magnified images of the white rectangle of (A) from multiphoton microscopy; (D) magnified histological image of the black rectangle of (B). (Scale bar: 100 μm.)

E14.5

Large scale of the multiphoton microscopy image of tooth germ in E14.5 was shown with the AF (Fig. 2A and C). In this and subsequent images, we used the green pseudocolor to denote AF and the blue pseudocolor to represent SHG signal. The image in Fig. 2C was the magnified image from Fig. 2A (white rectangle). The related histological analysis with H&E staining in the same area was shown in Fig. 2B which demonstrated the connection between tooth germ and epithelium. It revealed that the dental epithelium cells were started to be unfolded, and the mesenchymal cells were aggregated to form a dental papilla. A cap-like dental organ and dental papilla underneath was found. This indicated that the tooth germ was in cap stage and no SHG signal was found in this stage (Fig. 2A and C).

Fig. 2D shows the magnified image from Fig. 2B (black rectangle). Comparing the structures observed under multiphoton microscopy and histology analysis, the similarity of the outline of tooth germ images acquired using these two techniques were evident.

Postnatal Day 1 (P1)

The large scale of the multiphoton microscopy images of mice tooth germ in P1 were shown in Fig. 3A and C. The magnified image of Fig. 3A (white rectangle) was demonstrated in Fig. 3C. At this stage the shape of tooth germ was with bell-shape and initial formation of pre-dentin was shown with SHG (Fig. 3C). The related histological analysis with H&E staining showed the distribution of star-like stellate reticulum (SR) cells (Fig. 3B and D).

When comparing the structures observed under multiphoton microscopy and histological analysis, the images of tooth germ outline acquired using the two techniques are similar.

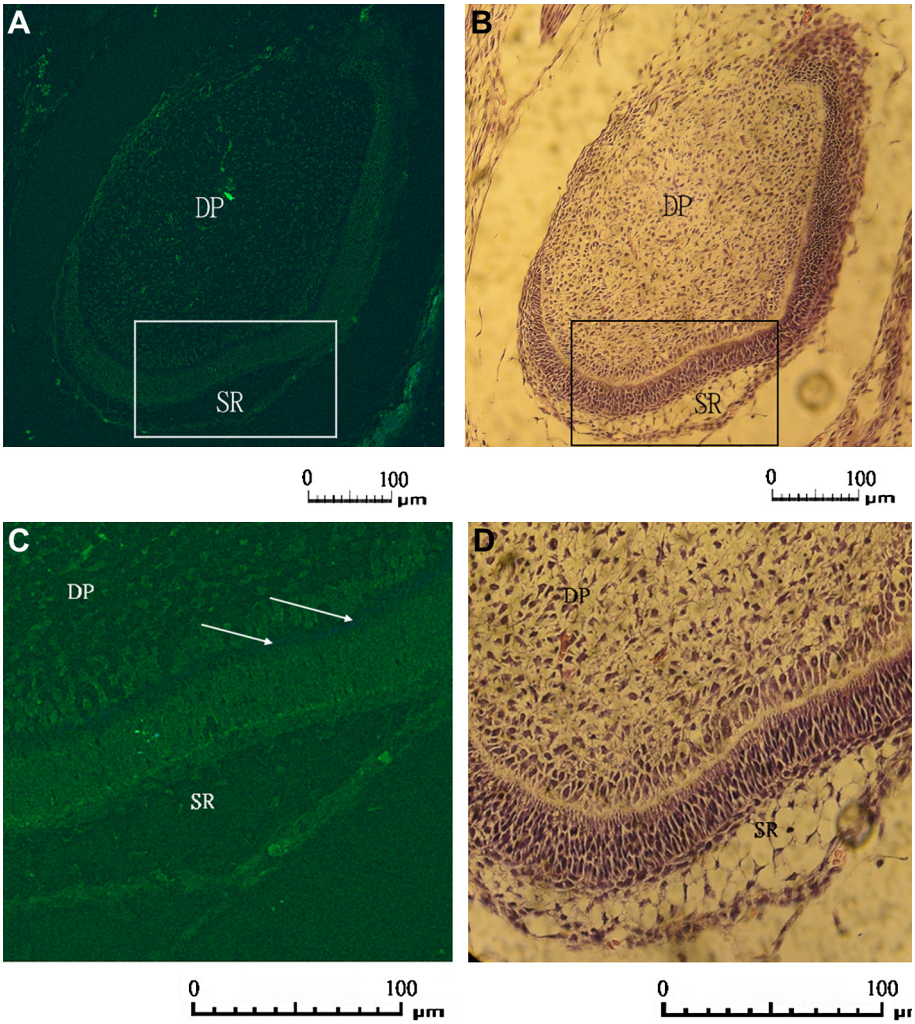

Figure 3 Comparison of images of tooth germ from Postnatal Day 1 (P1) mice investigated with multiphoton microscopy and histological analysis with hematoxylin and eosin staining under light microscopy. (A) Large scale of the multiphoton microscopy images revealed the shape of tooth germ was in a bell-shape; (B) correlated histological image from hematoxylin and eosin staining demonstrated star-like stellate reticulum; (C) magnified image of the white rectangle of (A). White arrows indicated slight SHG signal that was underneath the ameloblasts and indicated the initiation of predentin; (D) magnified image of the black rectangle of (B). (Scale bar: 100 μm.)

Postnatal Day 3 (P3)

The large scale of the multiphoton microscopy images of mice tooth germs in P3 were shown in Fig. 4A and C. It was found that in this stage, the shape of the enamel organ was changing to a bell shape that indicated that molar tooth germ was at bell stage. Dentin, predentin, enamel, ameloblasts, and odontoblasts were appeared clearly. From magnified image (Fig. 4C), the SHG signals can be detected in dentin and predentin, the alignment of enamel, odontoblasts and ameloblasts were also demonstrated.

For comparison, the large scale of histological images at this stage was shown in Fig. 4B. It was found that the inner enamel epithelium (IEE) and dental papilla (DP) were evident in the magnified image (Fig. 4D).

P5

The large-scale multiphoton microscopy images of mice tooth germ in this age of P5 were shown with the structure of enamel, dentin, and predentin (Fig. 5A). The magnified image from Fig. 5A was shown in Fig. 5C. In this stage, the layers of ameloblasts, enamel, dentin, predentin, and odontoblasts were recognized easily. The enamel, ameloblasts, and odontoblasts were shown with AF signal. The predentin was shown with SHG and dentin was shown both AF and SHG signals. In addition, the thickness of dentine was thicker than that of P3.

For comparison, histological images of the similar area correlated with the images of multiphoton microscopy were shown in Fig. 5B and D.

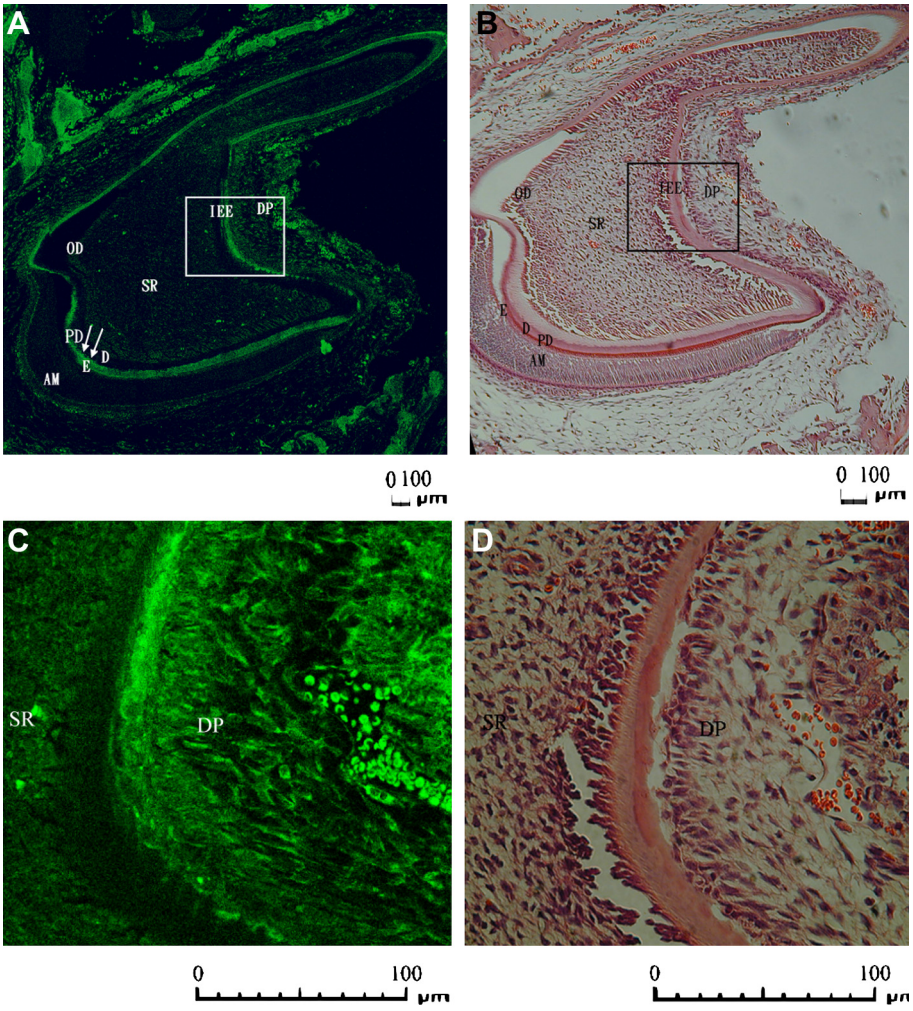

Figure 4 Comparison of images of tooth germ from Postnatal Day 3 (P3) mice investigated with multiphoton microscopy and histological analysis with hematoxylin and eosin staining under light microscopy demonstrated the tooth germ was in bell stage. (A) Large scale of the multiphoton microscopy images revealed that the shape of tooth germ with a bell shape indicated that molar tooth germ was in bell stage. The predentin was shown with SHG signal (white arrows) while enamel, ameloblasts and odontoblasts were appeared with AF clearly. The dentin was shown with both autofluorescence and SHG signals; (B) correlated histological image from hematoxylin and eosin staining in the same area of (A); (C) magnified image of the white rectangle of (A) indicated the alignment of ameloblasts, enamel, dentin, and odontoblasts; (D) magnified image of the black rectangle of (B) demonstrated the inner enamel epithelium, predentin, ameloblast, dental papilla, enamel, dentin, and odontoblasts. (Scale bar: 100 μm.)

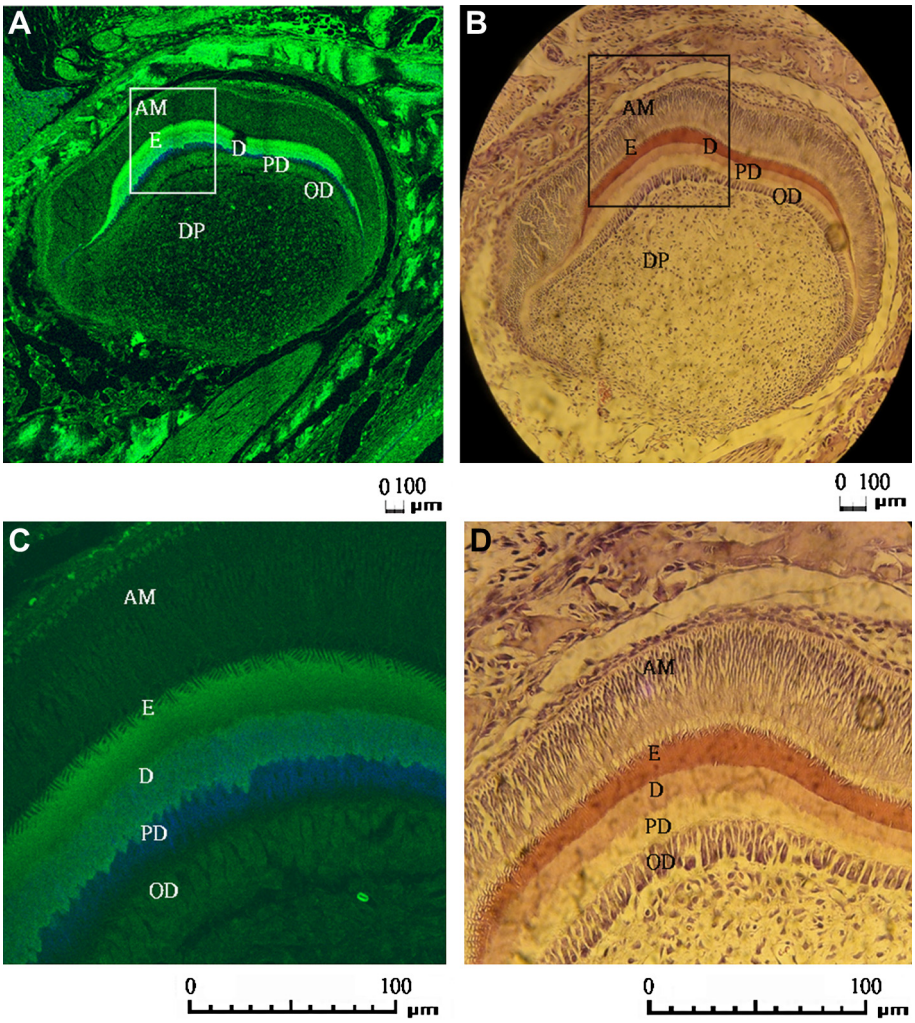

Figure 5 Comparison of images of tooth germ from postnatal day 5 (P5) mice investigated with multiphoton microscopy and histological analysis with hematoxylin and eosin staining observed under light microscopy. (A) Large scale of the multiphoton microscopy images demonstrated the structure of enamel, dentin, and predentin; (B) correlated histological image from hematoxylin and eosin staining in the same area of (A); (C) magnified image of the white rectangle of (A) indicated the morphology of ameloblasts and odontoblasts. The layers of ameloblasts, enamel and odontoblasts were shown with autofluorescence and structure of pre-dentin was shown with SHG. The structure of dentin was shown with both AF and SHG. Besides, dentin was thicker than that of P3. (D) The magnified image of the black rectangle of (b) demonstrated the alignment of ameloblast, predentin, dental papilla, enamel, dentin, and odontoblasts. (Scale bar: 100 μm.)

Fig. 5D showed the magnified images from Fig. 5B (black rectangle). It was found that the morphological similarity of the outline of tooth germ in the images acquired using the two techniques are evident.

P7

The large-scale multiphoton microscopy image of mice tooth germ was shown in Fig. 6A. Initiation of multiple cusps formation was found with evident structures of enamel, dentin, and predentin. Fig. 6C showed the magnified image from Fig. 6A.

For comparison, histology images of the similar area correlated with the images of multiphoton microscopy were shown in Fig. 6B and D. Morphology of ameloblasts and odontoblasts were demonstrated in Fig. 6D, which was magnified image from Fig. 6B. It was found that morphologies of the outline of tooth germs from the images acquired by using two different techniques were similar.

From our study, the stage of tooth germ development was found to be matched with the previous researches.[14–16] The mice tooth germ at E14.5 was in cap stage and at the age of P1 to P3 were in bell stage, and during P5 to P7 were in the late bell stage.

Discussion

For conventional study, histological staining is the most popular method to observe the structure of tooth development. In order to get the images of different stages of tooth growth, the tissue blacks should be prepared via a series of processes such as fixed by using chemical solution, decalcified, and then sliced for investigation. However, those processes of decalcification may loose the

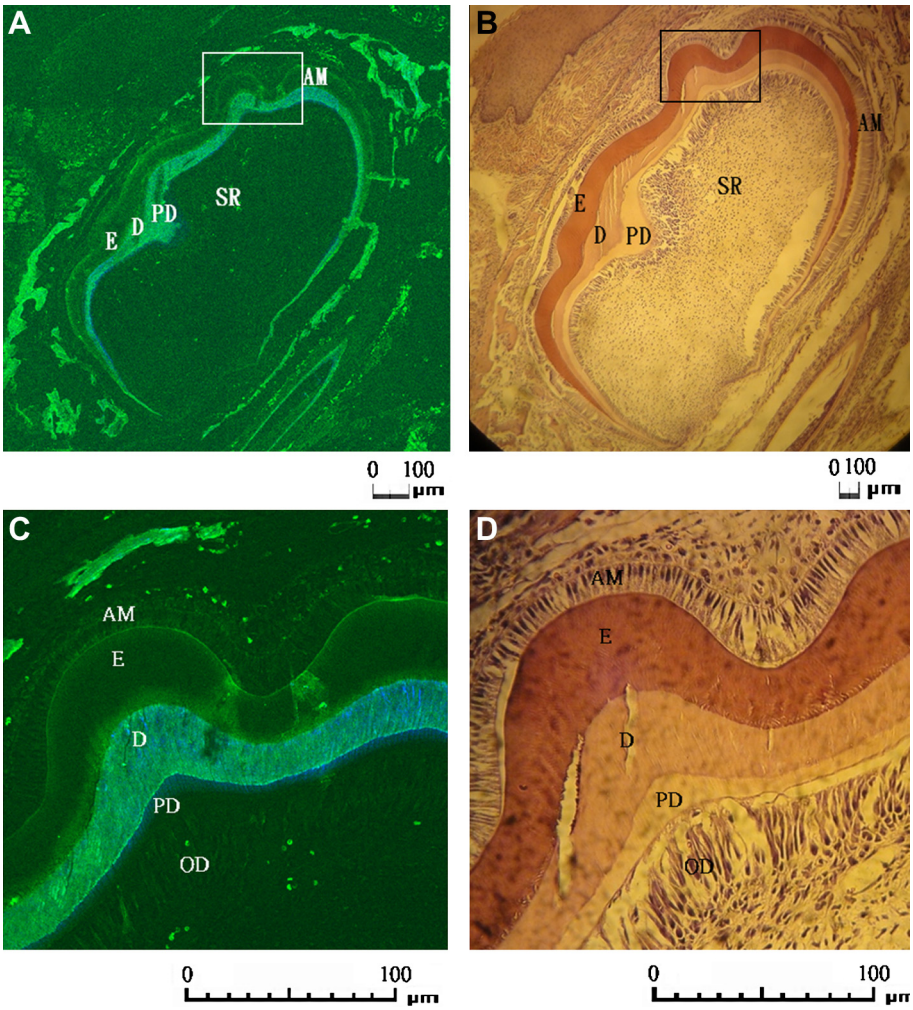

Figure 6 Comparison of images of tooth germ from postnatal day 7 (P7) mice investigated with multiphoton microscopy and histological analysis with hematoxylin and eosin staining observed under light microscopy. (A) Large scale of the multiphoton microscopy images demonstrated the initiation of multiple cusps formation; (B) correlated histological image from hematoxylin and eosin stain in the same area of (A) demonstrated the structure of enamel, dentin, predentin, odontoblasts, and stellate reticulum; (C) magnified image of the white rectangle of (A) demonstrated the alignment of ameloblasts, enamel, dentin, predentin, and odontoblasts; (D) magnified image of the black rectangle of (B). The images of tooth structure and morphology acquired by using two different methods were comparable. The blue color indicated SHG appeared in predentin. (Scale bar: 100 μm.)

information about the stages of mineralization during tooth development. In this study, different stages of tooth development were imaged by using multiphoton microscopy and were compared with histological staining; we build up a novel database of tooth development images with multiphoton microscopy.

According to our results, we found that pre-dentin can be imaged by multiphoton SHG signal, whereas other structures such as enamel, inner enamel epithelium (IEE), outer enamel epithelium (OEE), stellate reticulum (SR), odontoblast, and ameloblast can be imaged by multiphoton AF signals. The structure of dentin was shown with both AF and SHG, which were expressed the same with previous report.[12] The signal with SHG is important which may indicate the initiation stage of bell stage and this is a novel study to use multiphoton microscopy for investigating the structure of tooth germs development. From previous study, development of tooth in mice is obvious after mice were born and it is worthy to observe the development of tooth germ in postnatal mice.[15]

As reported previously, multiphoton laser microscopy has its limitation for imaging about only hundreds of microns in depth.[17] The depth is not very deep, but it is enough for our study to observe the details of tooth germ, especially for embryonic stage.

In this study, database of images of developing tooth germs in multiphoton microscopy was set up and compared with histological images. We proved that the images from multiphoton microscopy could be identified comparing to the histological images. It is worthy to design a miniature culture system, to be located on the multiphoton microscope, with thermal control and the oxygen and carbon dioxide supply for investigating the dynamic development of tooth germ. We suggested that by using multiphoton

microscopy, dynamic images of developing tooth germs could be acquired and worthy for application in the further study of tooth regeneration.

Conclusion

The database of images of developing tooth germ in multiphoton microscopy was set up in this study. The outline of developing tooth germ in different stage can be identified and compared with histological images. The predentin can be imaged with the SHG signal that is important to indicate the initiation of bell stage. We conclude that multiphoton fluorescence microscopy is worthy for further appliance in the study of tooth regeneration.

Acknowledgments

The authors thank the Core Facility from NTUH Medical Research Center for assistance in multiphoton laser fluorescence microscope and the financial support from National Science Council (NSC 97-2314-B-002-102-MY3 and NSC 99-2815-C-002-002-B). We also thank the *Molecular Center Core Facility and Research Center for Developmental Biology and Regenerative Medicine*.

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